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Detection of viral RNA from paraffin-embedded tissues after prolonged formalin fixation

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ABSTRACT

Background: Isolating amplifiable RNA from formalin-fixed, paraffin-embedded (FFPE) tissues is more difficult than isolating DNA because of RNases, chemical modification of the RNA, and cross-linking of nucleic acids and proteins. Tissues containing infectious disease agents that require biosafety level (BSL)-3 and -4 necessitate fixation times of 21 and 30 days, respectively.
Objective: To improve procedures for extracting RNA from these FFPE tissues and detect the RNA with the more sensitive TaqMan®-based reverse transcriptase (RT)-PCR.
Study design: Through a single modification of a commercially available kit, we were able to extract amplifiable RNA and detect West Nile virus (WNV), Marburg virus (MARV), and Ebola virus (EBOV)-infected tissues using TaqMan® assays.
Results: Formalin fixation results in an approximately 2 $\log_{10}$ reduction in detection limit when compared to fresh tissues. Increasing proteinase K digestion (24 h) improved extraction of amplifiable RNA from FFPE tissues. The TaqMan® results were comparable to more traditional detection results such as virus isolation.
Conclusion: This improved extraction procedure for obtaining RNA combined with the TaqMan® RT-PCR assays permit retrospective and prospective studies on FFPE tissues infected with BSL-3 and -4 pathogens.
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1. Introduction

PCR analysis of tissues is used frequently for detecting and identifying infectious disease agents because it provides improved sensitivity compared to other techniques, such as immunohistochemistry (IHC) and in situ hybridization. Traditionally, fresh or frozen tissue is used as a source of RNA or DNA, which limits PCR analysis to prospective studies. Recently, DNA extraction methods of formalin-fixed, paraffin-embedded (FFPE) tissues and PCR analysis have become possible.[1,2] However, similar analyses with RNA are problematic due to degradation by autologous RNases and the fixation and paraffin-embedding procedures. Extracting RNA from FFPE tissues is more difficult, requiring several days and the use of complex extraction methods. After extraction, the RNA is severely degraded to fragments averaging 100–300 nucleotides long.[3–5] In addition, formalin fixation modifies RNA by adding methylol groups ($-CH_2OH$) to nucleotides[6] which can interfere with reverse transcription of RNA that reduces PCR efficiency.

TaqMan® assays are real-time quantitative RT-PCR assays that precisely and linearly measure amplicon accumulation during the exponential phase of the reaction by detecting the increase in fluorescent emission. Coupled with 75–150 bp amplicon sizes, TaqMan® RT-PCR is ideal for analyzing RNA from FFPE tissues.

Clinical tissue samples are routinely formalin-fixed for less than 24 h followed by paraffin embedding. At the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) standard operating procedures require tissues infected with BSL-3 and -4 bioagents to be fixed in 10% neutral-buffered formalin (NBF) for 21 (BSL-3) or 30 days (BSL-4) prior to molecular pathology studies. However, prolonged fixation is thought to result in irreversible modifications to the RNA.[6] Our objective was to improve procedures for extracting RNA from these extensively formalin-fixed tissues and subsequently detect the RNA with TaqMan® RT-PCR assays.

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14. ABSTRACT

Isolating amplifiable RNA from formalin-fixed, paraffin-embedded (FFPE) tissues is more difficult than isolating DNA because the potential for RNA degradation by RNases, chemical modification of the RNA by addition of methylol groups (-CH2OH), and cross-linking of nucleic acids and proteins during the fixation process. In our laboratories, these difficulties are increased when extended fixation times of 3 to 4 weeks are required for tissues infected with BSL-3 or -4 agents. Four commercially available kits with different nucleic acid isolation chemistries were evaluated for their ability to isolate RNA from FFPE West Nile virus-infected tissues.The quality of the extracted RNA was determined by using a fluorogenic 5' nuclease TaqManTM) PCR assay. A modification of the Paraffin Block RNA Isolation Kit that included an overnight proteinase K digestion was necessary to obtain amplifiable RNA from tissues formalin-fixed for 21 days. Extracting TaqManTM amplifiable RNA from Marburg- and Ebola virus-infected tissues, formalin-fixed for at least 30 days, further tested the modified extraction method. This improved extraction procedure for obtaining amplifiable RNA combined with the more sensitive and specific fluorogenic probe-based PCR assays will now permit retrospective and prospective studies on FFPE tissues infected with BSL-3 and -4 pathogens.

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2. Materials and methods

2.1. FFPE tissue samples

Brain tissue from suckling mice experimentally infected intracranially with WNV strain NY99, along with additional WNV strain NY99-infected FFPE brain, liver, spleen, and kidney tissues from American crows (*Corvus brachyrhynchos*) were taken during necropsy and fixed in 10% NBF for 21 days, processed routinely, and paraffin-embedded. Following proteinase K pretreatment, WNV antigen detection was done by incubation with a rabbit hyperimmune polyclonal serum against WNV and a peroxidase-conjugated secondary antibody. Color was developed with 3,3′-diaminobenzidine solution containing H_2O_2. MARV Musoke- and EBOV Zaire-infected liver tissue from experimentally infected cynomolgus macaques (*Macaca fascicularis*) was immersion-fixed in 10% NBF formalin for 30 days, processed routinely, and embedded in paraffin.[7,8]

2.2. RNA extraction

Twenty-micrometer sections were deparaffinized at 25 °C with three washes in Xyless II (Val Tech Diagnostics, Brackenridge, PA) followed by three washes in 100% ethanol and air-dried for 5 min at room temperature. RNA was isolated using either the Paraffin Block RNA Isolation Kit (Ambion, Austin, TX) or the Optimum™ FFPE RNA Isolation Kit with modifications of the manufacturer's instructions. To optimize RNA extraction for prolonged FFPE tissues, we investigated the following modifications: (1) increased the deparaffinization temperature to 65 °C; (2) increased the proteinase K final concentration to 2 mg/ml; (3) increased the proteinase K incubation time to 24 h; (4) increased the RNA pellet drying temperature to 70 °C; (5) resuspended the RNA pellet in Tris–HCl, pH 7.4.

2.3. RNA extraction efficiency

The efficiency of RNA extraction was investigated by comparing the fresh frozen tissue extract to the corresponding FFPE tissue extract. Mouse brains were mid-sagittally divided in half, one half was snap frozen in liquid nitrogen, and stored at −70 °C; the other half was fixed in 10% NBF formalin for 30 days and paraffin-embedded. Sections were cut from the paired frozen and fixed tissues and RNA extracted. The murine housekeeping RNA, GAPDH (TaqMan® Rodent GAPDH Control Reagents; Applied Biosystems, Foster City, CA) was detected using real-time PCR.

2.4. TaqMan® assays

Real-time RT-PCR was performed with Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ) in a one-step procedure. For each 50 μl reaction, one bead was combined with virus-specific primers and probe, 2.5 U of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA), and 5 μl of RNA. Probe and primer sets were published for WNV,[9] MARV,[10] and EBOV.[11] The WNV assay contained 0.25 μM of each primer, WN3′NC-forward and reverse, and 0.2 μM of WN3′NC probe, and 2.5 mM $MgCl_2$. The MARV assay contained 0.5 μM of each primer, MBGGP3-forward and reverse, 0.2 μM MBGGP3 probe, and 5 mM $MgCl_2$. The Ebola assay contained 0.5 μM of each primer, EBOGP1D-forward and reverse, 0.2 μM EBOGP1DZPrb probe, and 5 mM $MgCl_2$. RT-PCR was performed with an ABI PRISM 7700 Sequence Detection System instrument (Applied Biosystems). Thermocycling conditions were: 42 °C for 30 min and 95 °C for 2 min, followed by 45 cycles of 95 °C for 5 s, and 60 °C for 20 s.

3. Results

3.1. RNA extraction optimization

Attempts to extract amplifiable WNV RNA from FFPE tissues using the unmodified commercial kits were unsuccessful (data not shown). Only increasing the proteinase K digestion from 2 to 24 h resulted in the recovery of RNA that could be detected in the TaqMan® assay.

3.2. RNA extraction efficiency

Detection of GAPDH RNA extracted from paired frozen and fixed tissues by real-time PCR indicated FFPE tissues had a shift of 6–8 cycles in C_T value (later) than the corresponding frozen tissues (Table 1), indicating a significant decrease in extracted amplifiable RNA. Since the frozen and FFPE tissue sections were matched, we assumed that the amount of RNA present in each type of sample was equivalent; therefore the difference could be due to either RNA modifications or PCR inhibitors in the FFPE tissues. Inhibition occurs when extraction procedures fail to remove substances that interfere with the RT and/or PCR. To detect inhibition we added samples of the extracted RNA to an exogenous internal positive control (IPC)[12] and compared their C_T values. A shift of 3.3 C_T cycles is equivalent to a one-log loss of detection limit and is considered inhibitory. One FFPE tissue section from each of two mice (Table 1; Mouse 2A and 3A) failed to produce a PCR signal compared to other sections from the same mice. Since no inhibition was observed, the reason may be the result of inefficient extraction, RNases, or some other negative PCR effect.

3.3. RNA isolations from FFPE tissue samples

WNV-infected crow brain, liver, kidney, and spleen tissues tested by the modified RNA isolation protocol were all positive by the TaqMan® assay. TaqMan® compared favorably to the IHC and virus-isolation results (Table 2). Tissues that had the greatest IHC reactivity and highest viral titers generally also had the lowest C_T values (greater amounts of viral RNA). As expected, uninfected tissue controls were negative by TaqMan® analysis. Interestingly, IHC of crow G56 brain tissue failed to demonstrate the presence of viral antigen in brain tissue; however, viral RNA could be readily detected after extraction and TaqMan® analysis. This could be explained by the TaqMan® assay detecting viral RNA before it is translated into viral proteins. Unfortunately, no tissues were collected for virus isolation.

Table 1
Efficiency of RNA extraction from formalin-fixed, paraffin-embedded (FFPE) tissues compared to fresh frozen tissues. Sections of mouse brain tissue were cut and RNA extracted from paired fixed and frozen preparations. The murine house-keeping gene, GAPDH, was detected using real-time PCR.

Sample	Crossing threshold (C_T) value	
	Frozen	FFPE
Mouse 1A	21.86	27.58
1B	20.77	28.55
1C	20.71	27.01
Mouse 2A	22.34	>45[a]
2B	21.63	27.35
2C	21.71	31.48
Mouse 3A	23.31	>45
3B	21.16	28.21
3C	21.38	26.47

[a] C_T value ≥ 45 is negative.

Table 2
TaqMan® analysis (C_T value), immunohistochemistry reactivity, and viral titers for formalin-fixed, paraffin-embedded (FFPE) tissues from wild-caught American crows (*Corvus brachyrhynchos*) experimentally infected with West Nile virus, NY99.

Sample[a]	C_T value	IHC[b]	Viral titers[c]
2A	32.65	3	5.30
2B	32.57	3	5.30
2C	32.23	3	5.30
2H	26.26	4–7	4.00–5.70
4A	35.74	2	3.48
4B	33.83	2	3.48
4C	32.68	2	3.48
4H	24.87	4	4.00–5.70
14A	32.33	3	5.70
14B	30.44	3	5.70
14C	34.61	3	5.70
14G	26.54	3–6	2.00–5.18
18C	29.97	4	4.00
18H	24.96	3–7	4.70–5.70
G56A	24.65	0	ND
G56B	25.27	0	ND
G56C	24.85	0	ND
G56I	20.28	4–9	ND
Positive	31.28	ND[d]	ND
Negative	>45[e]	ND	ND

[a] Samples A, B, and C represent brain tissue each in a separate FFPE block. Samples G, H, and I represent liver, kidney, and spleen tissue combined in a single block; therefore combined viral titers are compared to a single real-time PCR result.
[b] IHC, immunohistochemistry results are recorded as immunoreactivity graded on a scale of 0–10, 0 being no immunoreactivity and 10 being very intense reactivity.
[c] The viral titer was expressed as WNV $\log_{10}$ PFU/ml of a 10% tissue suspension.
[d] Not determined.
[e] C_T value $\geq$ 45 is negative.

With MARV, the TaqMan® assay detected virus-specific RNA in each infected liver sample (Table 3). Because the nonhuman primates (NHP) were sampled after they exhibited clinical signs, the viral titers were very high, approximately 9.0 $\log_{10}$/g of tissue. In contrast, the NHP in the EBOV virus study were infected on day 0 and three animals were necropsied on consecutive days 2, 3, 4, 5, and 6. Liver sections from two animals per day were available for testing. EBOV RNA was detected by the TaqMan® assay and increased each day until leveling off at days 5 and 6 (Table 4). Uninfected NHP liver tissue controls were negative. TaqMan® assay results compared favorably with the virus-isolation data; EBOV RNA could not be detected at day 2 post-infection and was present in only one animal on day 3. By day 4, virus RNA was demonstrated in each animal tested until the end of the study.

Table 3
TaqMan® analysis (C_T value) and viral titers for formalin-fixed, paraffin-embedded (FFPE) liver tissues from cynomolgus macaques (*Macaca fascicularis*) experimentally infected with Marburg virus, Musoke.

Sample	C_T value	Viral titer[a]
C163A	25.83	9.90
D185A	20.97	8.93
14880	21.61	9.85
5711	24.98	9.40
5706	23.75	9.65
5695	22.69	9.20
Positive[b]	20.41	ND[c]
Negative[d]	>45[e]	ND

[a] The viral titer was expressed as Marburg Musoke virus $\log_{10}$ PFU/g.
[b] Positive control was Marburg virus, Musoke-infected cynomolgus macaque liver tissue.
[c] Not done.
[d] Negative control was uninfected cynomolgus macaque liver tissue.
[e] C_T value $\geq$ 45 is negative.

Table 4
TaqMan® analysis (C_T value) and viral titers for formalin-fixed, paraffin-embedded (FFPE) liver tissues from cynomolgus macaques (*Macaca fascicularis*) experimentally infected with Ebola virus, Zaire.

Sample	Day postinoculation	C_T value	Viral titer[a]
201196	2	>45[b]	0
201197	2	>45	0
201199	3	35.02	4.83
201200	3	>45	0
201203	4	31.06	6.41
201204	4	25.75	8.11
201205	5	26.36	7.73
201206	5	24.57	8.10
201209	6	24.41	8.03
201210	6	26.58	8.34
Negative	NA[c]	>45	NA

[a] The viral titer was expressed as Ebola Zaire virus $\log_{10}$ PFU/g.
[b] C_T value $\geq$ 45 is negative.
[c] Not applicable.

4. Discussion

Formalin is the most commonly used fixative in pathology laboratories. Extracting DNA from FFPE tissues has become routine[13] but RNA extraction remains difficult and inefficient, due to degradation or modification of the RNA. Formalin fixation-induced cross-linking between proteins and DNA or RNA is probably the most significant result of formalin fixation[13–15] and is most likely the greatest obstacle for obtaining RT-PCR-amplifiable RNA. Cross-linking preserves cellular structure, but traps the nucleic acids in tissues, making their extraction more difficult[16]; disrupts the chain elongation of the nascent DNA by interfering with primer extension of the RT[14]; and modifies RNA by adding mono-methylol ($-CH_2OH$) groups to all four bases, interrupting reverse transcription.[6] Extraction efficiency of amplifiable RNA from a FFPE tissue is inversely proportional to the fixation time.[17–19] Therefore, tissues containing pathogens requiring BSL-3 and -4 containment and extended formalin fixation to ensure inactivation, pose a significant challenge for the extraction of amplifiable RNA and use in PCR-based assays.

In this study, we tested commercially available RNA extraction kits for their ability to purify amplifiable RNA. Only extending the proteinase K digestion from 2 to 24 h with the Ambion and Optimum Kits resulted in successful isolation of amplifiable RNA. The Ambion Kit included a proteinase K/GITC lysis, an acid–phenol:chloroform extraction, and RNA precipitation. Lewis et al. found this method to be the most successful extraction procedure for recovering RNA from FFPE tissues.[13] The Optimum Kit that replaced the phenol:chloroform extraction/RNA precipitation with an elution-based filter cartridge produced comparable results. A 24 h proteinase K digestion improved amplifiable RNA extraction significantly.[1,20,21] Proteinase K is thought to release RNA trapped in the cross-linked protein–nucleic acid matrix, permitting DNA/RNA isolation by acid–phenol:chloroform or filter cartridge. Our assumption, is the extended fixation times required for tissues infected with BSL-3 and -4 agents results in more extensive cross-linking, which required longer digestion to release RNA. Jackson et al. found that FFPE tissues digested in proteinase K for 5 days produced considerably more RNA.[1] However, Jiang et al. found proteinase K treatment unnecessary to release RNA trapped by formalin fixation but the 0.1 mg/ml concentration may have

been insufficient.[22] Alternatively, they found sonication and DNase digestion to be critical.

Cross-linking and modification of RNA by formalin fixation limits the size of the cDNA product produced and ultimately the amplicon length. TaqMan® assays are ideally suited for detecting RNA in FFPE tissues because they require only small amounts of template and produce small amplicons.[23–25] Comparison of TaqMan® GAPDH RNA detection in fresh and FFPE paired tissues resulted in an increased C_T value of six to eight cycles for fixed tissues which represents a decrease of approximately $2 \log_{10}$ of sensitivity (Table 1). Presumably this decrease is the result of formalin-induced RNA modifications. The WNV TaqMan® assay was used to monitor the optimization of the RNA extraction modifications.[9] C_T values from this sensitive assay compared well with other measures of WNV infection; generally decreasing as the number of virus-infected cells and viral titers increased (Table 2). TaqMan® assays of MARV- and EBOV-infected FFPE tissues yielded similar results (Tables 3 and 4). The close comparisons between viral titers and the TaqMan® C_T values suggest that the assay is at least as sensitive as virus isolation, the virological gold standard.

Clearly, we have demonstrated that amplifiable RNA can be recovered from FFPE tissues after prolonged fixation in formalin. Utilizing the TaqMan® assay for detecting RNA overcomes many of the difficulties that are associated with formalin-fixed tissues. As the number of high biocontainment laboratories increases, greater numbers of investigators will be confronted with the challenge of extracting amplifiable RNA from tissues fixed in 10% NBF for prolonged periods of time and its use in newer and more powerful assays developed since the original tissues were collected.[26] The extensive archives of FFPE tissues at USAMRIID, from a wide variety of animal studies with biological agents requiring high-level biocontainment, will allow retrospective studies to be done, thus saving time, valuable animals, resources, and laboratory space, as well as decreasing the potential exposure of laboratory workers to hazardous biological agents.

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References

1. Jackson DP, Lewis FA, Taylor GR, Boylston AW, Quirke P. Tissue extraction of DNA and RNA and analysis by the polymerase chain reaction. *J Clin Pathol* 1990;**43**:499–504.
2. Sarkar FH, Crissman JD. Detection of human papilloma virus DNA sequences by polymerase chain reaction. *BioTechniques* 1990;**9**:180–5.
3. Abe K, Tani M, Edamoto Y, Hayashi S, Saito T, Kurata T. Detection of hepatitis C virus genome in paraffin-embedded tissues by nested reverse transcription polymerase chain reaction. *Int Hepatol Commun* 1994;**2**: 352–7.
4. Soguero C, Ribalta T, Campo E, Sanchez-Tapies J, Saiz J, Bruguera M. Detection of hepatitis C virus RNA in more than 20-year-old paraffin-embedded liver tissue. *Lab Invest* 1999;**79**:365–6.
5. Stanta G, Schneider C. RNA extracted from paraffin-embedded human tissues is amenable to analysis by PCR amplification. *BioTechniques* 1991;**11**: 304–8.
6. Masuda N, Ohnishi T, Kawamoto S, Monden M, Okubo K. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. *Nucleic Acids Res* 1999;**27**: 4436–43.
7. Wilhelmsen CL, Hevey M. Histomorphologic and immunohistochemical characterization of experimental Marburg virus infection in cynomolgu macaques (*Macaca fascicularis*). *Vet Pathol* 2001;**38**:590.
8. Geisbert TW, Hensley LE, Larsen T, Young HA, Reed DS, Geisbert JB, et al. Pathogenesis of Ebola hemorrhagic fever in cynomolgus macaques: evidence that dendritic cells are early and sustained targets of infection. *Am J Pathol* 2003;**163**:2347–70.
9. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, et al. Rapid detection of west nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. *J Clin Microbiol* 2000;**38**:4066–71.
10. Gibb TR, Norwood Jr DA, Woollen N, Henchal EA. Development and evaluation of a fluorogenic 5′-nuclease assay to identify Marburg virus. *Mol Cell Probes* 2001;**15**:259–66.
11. Gibb TR, Norwood Jr DA, Woollen N, Henchal EA. Development and evaluation of a fluorogenic 5′ nuclease assay to detect and differentiate between Ebola virus subtypes Zaire and Sudan. *J Clin Microbiol* 2001;**39**: 4125–30.
12. Hartman LJ, Coyne SR, Norwood Jr DA. Development of a novel internal positive control for Taqman based assays. *Mol Cell Probes* 2005;**19**:51–9.
13. Lewis F, Maughan NJ, Smith V, Hillan K, Quirke P. Unlocking the archive-gene expression in paraffin-embedded tissue. *J Pathol* 2001;**195**:66–71.
14. Park YN, Abe K, Li H, Hsuih T, Thung SN, Zhang DY. Detection of hepatitis C virus RNA using ligation-dependent polymerase chain reaction in formalin-fixed, paraffin-embedded liver tissues. *Am J Pathol* 1996;**149**: 1485–91.
15. Rupp GM, Locker J. Purification and analysis of RNA from paraffin-embedded tissues. *BioTechniques* 1988;**6**:56–60.
16. Chalkley R, Hunter C. Histone–histone propinquity by aldehyde fixation of chromatin. *Proc Natl Acad Sci USA* 1975;**72**:1304–8.
17. Ben Ezra J, Johnson DA, Rossi J, Cook N, Wu A. Effect of fixation on the amplification of nucleic acids from paraffin-embedded material by the polymerase chain reaction. *J Histochem Cytochem* 1991;**39**:351–4.
18. Bresters D, Schipper ME, Reesink HW, Boeser-Nunnink BD, Cuypers HT. The duration of fixation influences the yield of HCV cDNA-PCR products from formalin-fixed, paraffin-embedded liver tissue. *J Virol Methods* 1994;**48**:267–72.
19. Koopmans M, Monroe SS, Coffield LM, Zaki SR. Optimization of extraction and PCR amplification of RNA extracts from paraffin-embedded tissue in different fixatives. *J Virol Methods* 1993;**43**:189–204.
20. Coombs NJ, Gough AC, Primrose JN. Optimisation of DNA and RNA extraction from archival formalin-fixed tissue. *Nucleic Acids Res* 1999;**27**:e12.
21. Hicks DG, Stroyer BF, Teot LA, O'Keefe RJ. In situ hybridization in skeletal tissues utilizing non-radioactive probes. *J Histotechnol* 1997;**20**:215–24.
22. Jiang YH, Davidson LA, Lupton JR, Chapkin RS. A rapid RT-PCR method for detection of intact RNA in formalin-fixed paraffin-embedded tissues. *Nucleic Acids Res* 1995;**23**:3071–2.
23. Godfrey TE, Kim SH, Chavira M, Ruff DW, Warren RS, Gray JW, et al. Quantitative mRNA expression analysis from formalin-fixed, paraffin-embedded tissues using 5′ nuclease quantitative reverse transcription-polymerase chain reaction. *J Mol Diagn* 2000;**2**:84–91.
24. Goldsworthy SM, Stockton PS, Trempus CS, Foley JF, Maronpot RR. Effects of fixation on RNA extraction and amplification from laser capture microdissected tissue. *Mol Carcinogen* 1999;**25**:86–91.
25. Specht K, Richter T, Muller U, Walch A, Werner M, Hofler H. Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. *Am J Pathol* 2001;**158**:419–29.
26. Enserink M. Virology. The boom in biosafety labs. *Science* 2000;**288**:1320–2.